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Roles of SNAP-25 isoforms in activity-dependent long-term synaptic plasticity

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Roles of SNAP-25 isoforms in activity-dependent long-term synaptic plasticity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to the memory of Maisoon …..

beloved sister, mother, daughter and to her altruistic life …

ABSTRACT

SNARE proteins, SNAP-25, syntaxin 1A and VAMP2 constitute the functional units which join together to form the core SNARE complex. The SNARE complex carries out the vital function of membrane fusion of intracellular vesicles with plasma membranes, leading to the release of neurotransmitters in brain neuronal circuits and of hormones in endocrine glands. SNAP-25 exists as two alternatively spliced isoforms, resulting in two similar but distinct proteins, SNAP-25a and SNAP-25b. The distribution of these two proteins in brain and periphery are regulated developmentally. In this thesis, the focus has been on SNAP-25 in hippocampus. We evaluated the roles of SNAP-25 isoforms, and SNAP-25 mutants in activity-dependent long-term potentiation (LTP) and depression (LTD) at hippocampal Schaffer collateral-CA1 synapses.

We utilized gene targeted mouse models, *i)* the first only expressing SNAP-25a (the SNAP-25b-deficient mouse) and *ii)* the second having a mutated C-terminus of SNAP-25 (the SNAP-25Δ3 mouse), to investigate alterations in synaptic plasticity. SNAP-25b-deficient mice displayed a reduced magnitude of LTP at Schaffer collateral-CA1 synapses and an enhanced magnitude of LTD at similar synapses at similar age. These mice exhibited abnormalities in basal synaptic transmission, short-term synaptic plasticity (STP) and faster neurotransmitter release kinetics. Abnormalities in synaptic transmission were evident as deficits in learning and memory formation in a behavioral task of active avoidance. Mutations in the C-terminus of SNAP-25 reduce the ability of inhibitory Gβγ subunits to interact with SNAP-25, and we show here that SNAP-25Δ3 mice exhibit enhanced LTP at Schaffer collateral-CA1 synapses.

Lack of SNAP-25b causes hyperinsulinemia and, combined with Western diet, results in a diabetic phenotype. We investigated if a metabolic phenotype triggered by SNAP-25b-deficiency, or Western diet alone, affected higher cognitive functions of the brain. SNAP-25b-deficient mice and wild type mice with diet-induced metabolic syndrome performed poorly in brain region-specific behavioral tasks. Proteins quantification in the specific brain areas revealed changes in the expression levels of the SNARE proteins.

In conclusion, SNAP-25a and SNAP-25b play specialized and different roles in synaptic transmission. The roles of SNAP-25b appear to be more suited to a mature brain with stronger synaptic connectivity, and the work in this thesis clarifies the presynaptic contributions of the SNAP-25 isoforms to activity-dependent synaptic plasticity.

LIST OF SCIENTIFIC PAPERS

I. **Muhammad Irfan**, Katisha R. Gopaul, Omid miry, Tomas Hökfelt, Patric K. Stanton, Christina Bark. SNAP-25 isoforms differently regulate synaptic transmission and long-term synaptic plasticity at central synapses. *Sci Rep. 2019, 9(1):6403. doi: 10.1038/s41598-019-42833-3.*

II. **Muhammad Irfan**, Zack Zurawski, Heidi Hamm, Christina Bark, Patric K. Stanton. Disabling Gβγ-SNAP-25 interaction in gene targeted mice results in enhancement of LTP at Schaffer collateral-CA1 synapses in hippocampus. *Neuroreport. 2019 May 14. doi: 10.1097/WNR.0000000000001258.*

III. Katisha R. Gopaul, **Muhammad Irfan**, Omid Miry, Linnea R. Vose, Alexander Moghadam, Tomas Hökfelt, Christina Bark, Patric K. Stanton. Developmental time course of SNAP-25 isoforms regulation of hippocampal long-term synaptic plasticity and hippocampus-dependent learning. (*Manuscript*)

IV. **Muhammad Irfan**, Ismael Valladolid-Acebes, Tomas Hökfelt, Christina Bark. Effects of SNAP-25b-deficiency and Western diet intervention on brain SNARE proteins and behavior. (*Manuscript*)

PUBLICATIONS NOT INCLUDED IN THIS THESIS

1 **Muhammad Irfan**, Teresa Daraio, Christina Bark. SNAP-25 puts SNAREs at center stage in metabolic disease. *Neuroscience 2018 pii: S0306-4522(18)30510-4. doi: 10.1016/j.neuroscience.2018.07.035*

CONTENTS

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BoNT	Botulinum neurotoxins
CA1	Cornu ammonis area 1
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
CD	Control diet
CNS	Central nervous system
CRB	Cerebellum
DG	Dentate gyrus
EC	Entorhinal cortex
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
GABA	Gamma-aminobutyric acid
GC	Granule cell
GIRK	G protein-coupled inwardly rectifying K^+ channel
GPCR	G protein-coupled receptor
HC	Hippocampus
HFS	High frequency stimulation
LFS	Low frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MF	Mossy fibers
mGluR	Metabotropic glutamate receptor
NMDA	*N*-methyl-D-aspartate
NSF	*N*-ethylmaleimide sensitive factor
PI3K	Phosphatidylinositol 3-kinase
PFC	Prefrontal cortex
PKA/C	Protein kinase A/C
PLC	Phospholipase C

PPF	Paired-pulse faciliation
*P*r	Release probability
PSD	Postsynaptic density
RRP	Readily releasable pool
shRNA	Short/small hairpin ribonucleic acid
SNAP-25	Synaptosomsal associated protein of 25kDa
SNARE	Soluble *N*-ethylmaleimide sensitive factor Attachment protein REceptor
SPM	Synaptic plasticity memory
STP	Short-term plasticity
SV	Synaptic vesicle
TBS	Theta burst stimulation
VAMP	Vesicle-associated membrane protein
VGCCs	Voltage-gated Ca^{2+} channels
WD	Western diet
WT	Wild type
5-HT	5-hydroxytryptamine

1 SYNAPTIC PLASTICITY

1.1 A synapse and its ability to undergo a plastic change

A synapse refers to the micro space, which exists as a point of contact between two neurons in the brain. It was the pioneering histological work of Santiago Ramón y. Cajal in the 1890's, a Spanish neuroanatomist, who utilized staining methods developed by Camillo Golgi to stain and make drawings of the neurons of the central (CNS) and peripheral nervous sytem. He showed for the first time, among other things, that the neurons are not continuous extensions of each other like an electrical cable but they lay adjacent and separately to each other[1]. An English neurophysiologist, Charles S. Sherrington, coined the word 'synapse'[2]. Camillo Golgi and Santiago Ramón y. Cajal shared the Nobel Prize in Physiology and Medicine in 1906 for their work and up until today, Cajal is credited to have laid the foundation of modern neuroscience.

Ensuing research in to the cellular architecture and organization of neurons in brain led to the differentiation of a synapse into a presynaptic and a postsynaptic locus with synaptic cleft spanning an area of approximately 20-25nm[3,4]. The presynaptic area contains a dense protein rich zone, holding vesicles filled with neurotransmitters, which are released in to the synaptic cleft when a neuron is sufficiently stimulated/excited. The neurotransmitters diffuse across the synapse and bind to the receptors on the postsynaptic neuron, leading to excitation (e.g. with the neurotransmitter glutamate[5]) or inhibition (e.g. with the neurotransmitter GABA[6]) of the postsynaptic neuron. Across the brain, neurons are arranged in a fashion to what can be analogous with electrical circuits, with information flowing from one neuron to the other through synapses.

The notion that synapses are not merely a static point of contact between neighboring neurons and, instead, a dynamic entity, the strength of which can change with the activity of neurons, was first theoretically conceived by a Canadian neuropsychologist, Donald Hebb[7]. He proposed the 'Hebbian Rule or theory' of synaptic efficacy in his landmark book 'The Organization of Behavior' in 1949. According to the 'Hebbian Rule', if a neuron is active and persistently stimulates a neighboring neuron, it will lead to the strengthening of the connection between these two neurons, a phenomenon summarized as "neurons that fire together wire together"[7] The change in efficacy/strength of synaptic connection refers to the

plastic change. Depending on the duration for which the change lasts, synaptic plasticity can be short-term (msec) or long-term (min to days).

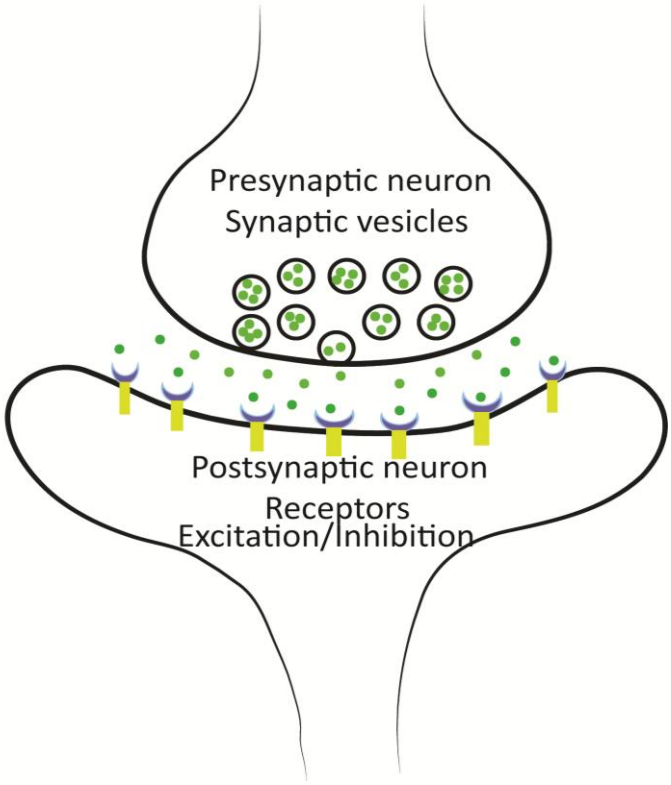

Figure 1: Schematic representation of the presynaptic and postsynaptic neuronal terminals in the central nervous system (CNS). The presynaptic locus contains neurotransmitter filled synaptic vesicles (SVs) and is rich in proteins, which help regulate the release of these neurotransmitters. The postsynaptic locus contains receptors for the neurotransmitters, and information is in this manner relayed from one neuron to the next via the synapse cleft.

1.2 Long-term potentiation (LTP) and long-term depression (LTD), Hebbian versus non-Hebbian forms of plasticity

Long-term potentiation (LTP) represents a physiological phenomenon in which the strength of a connection between two neurons in the brain gets stronger. The most well known reason for the change in synaptic strength is a high degree of neuronal activity, which makes the synapse stronger, and hence, the phenomenon is referred to as activity-dependent LTP. If a high degree of presynaptic stimulation or synchronous activity between the pre- and postsynaptic neuron leads to a stronger connection between neurons, it did not take the scientists long to figure out that a reduced or asynchronous activity will lead to a weakening of synaptic strength, a phenomenon referred to as long-term depression (LTD). The

distinction between activity- and non-activity dependent forms of synaptic plasticity is important. This is because after decades of research, it is now established that there are other forms of synaptic plasticity, which do not follow the Hebbian rule, but can be induced robustly in networks of neurons. Activity-dependent plasticity involving synaptic stimulation is classified as Hebbian plasticity, while the examples of non-Hebbian plasticity include homeostatic plasticity[8] or synaptic scaling[9,10]. Non-Hebbian forms of plasticity work in concert with Hebbian plasticity in neuronal networks and make the plastic changes more stable[11].

1.3 Discovery of long-term potentiation (LTP) and long-term depression (LTD)

The first empirical evidence of LTP was discovered by Terje Lømo and Tim Bliss in 1973, who showed that the strength of synapses could be potentiated for as long as 10 hours following a brief but intense tetanic stimulation[12]. They utilized anaesthetized rabbits to record the electrical activity from perforant path-granule cell synapses in the hippocampus. To express LTP, they used high frequency stimulus (HFS), which is still being applied in slightly different versions of LTP research. The basic principle of HFS for the expression of LTP in *ex vivo* brain slices involves injecting small bursts of constant current stimuli at approximately 100Hz frequency. The number of bursts may vary depending on the experimental setup. This is to mimic the high neuronal firing rate under conditions of intense activity, and as a result, when the presynaptic neuron is stimulated in an ordinarily fashion, the postsynaptic response is significantly larger, serving as evidence of Hebbian plasticity at the synapse. Heterosynaptic long-term depression (LTD) was discovered soon after[13], when scientists showed that synapses which did not receive HFS exhibited synaptic depression. It took relatively longer time to realize that prolonged low frequency stimuli (LFS) (1-3 Hz) can induce homosynaptic LTD [14,15]. Initial studies after the discovery of LTD, focused on the fact that LFS can reverse stable LTP[16], which was a correct finding. However, focus on the phenomenon of LTD was at the time relatively less than of LTP, until the 1990's.

1.4 Cellular and molecular changes associated with LTP and LTD postsynaptically

It may appear logical and straight forward to think that a plastic change at the synapse will be the result of both presynaptic (changes in neurotransmitter release rate) and postsynaptic (response/sensitivity to the neurotransmitter) changes, but it was not the case for a long time after the discovery of these phenomena[17,18]. In hindsight, researchers tracked the origin of controversy whether LTP was expressed pre- or postsynaptically to the initial lack of appropriate tools/methods for detecting presynaptic changes associated with LTP.

Initial studies pursuing the underlying mechanism of LTP showed that postsynaptic depolarization coupled with normal synaptic stimulation were enough to induce LTP even without HFS[19,20]. But with the advancements in pharmacological tools it became evident that the reason for this observation was that, in fact, *N*-methyl-D-aspartate (NMDA)-type glutamate receptors expressed postsynaptically are blocked by Mg^{2+} under normal hyperpolarized conditions and that this block is relieved when the postsynaptic neuron is depolarized[21,22]. Further studies showed that NMDA receptors conduct Ca^{2+} ions towards the inside when in the open state[23,24]. When postsynaptic Ca^{2+} was captured by EGTA, it blocked expression of LTP via HFS[25], hence, postsynaptic Ca^{2+} influx emerged as a necessary component for the expression of LTP, not necessarily via NMDA but through another glutamate receptor, called α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors[26,27]. Ca^{2+} recruits calcium/calmodulin-dependent kinase II (CamKII) which leads to its auto phosphorylation[28-30] and a series of events after the activation of this enzyme. CamKII modifies the cytoarchitecture of the postsynaptic area through engaging cytoplasmic actin[31,32]. This allows room for extra AMPA-type glutamate receptors to be inserted in the postsynaptic plasma membrane (locality), which are synthesized and constitutively secreted as well as captured from extra synaptic zones[33,34]. AMPA receptors have relatively faster kinetics compared to NMDA receptors and, when activated by glutamate, they conduct both Na^{+} and K^{+}. Unlike NMDA receptors they are not blocked by Mg^{2+} under hyperpolarizing conditions, hence are not voltage dependent. This leads to rapid postsynaptic depolarization in response to glutamate release and hence, even greater Ca^{2+} influx through NMDA receptors. This mechanism represents the general neural substrate for the postsynaptic component of LTP.

Ca^{2+} signaling inside the cell is incredibly diverse[35], and over the years, protein kinases other than CamKII have been shown to be activated via rises in intracellular $[Ca^{2+}]_i$. These include protein kinase A (PKA)[36-38], p42/44 mitogen-activated protein kinase (MAPK)[39], extracellularly regulated kinase (ERK)[39] and phosphatidylinositol 3-kinase (PI3K)[40]. These protein kinases influence gene transcription, which eventually leads to structural modifications suited for the induction and expression of LTP.

One would assume that the induction of LTD is associated with the opposite of what happens during LTP, but this is not quite the case. As it turned out, NMDA receptor activation and postsynaptic Ca^{2+} are important for induction of LTD as well[14]. Capturing postsynaptic Ca^{2+} via

the chelators EGTA or BAPTA blocked LTD[41]. As baffling as those earlier findings were, it was later shown that the key lies in abrupt massive increases of Ca^{2+} concentrations which happen during HFS and prolonged small increases of Ca^{2+} concentrations which occur during LFS. Persistent small rises in the intracellular Ca^{2+} concentration lead to reduced activation of CamKII because of the constraints on spatial availability of Ca^{2+}. This in turn activates protein phosphatases[42] (PP1, PP2B) which dephosphorylates AMPA receptors eventually leading to the depression of postsynaptic responses[43]. Blocking of those protein phosphatases abolished LTD[44].

NMDA and AMPA receptors are ionotropic receptors but LTD is not entirely dependent on NMDA receptors alone, and activation of metabotropic glutamate receptors (mGluRs) have also been shown to play a role in the induction of LTD[45,46]. mGluR receptors are G-protein coupled receptors (GPCRs) expressed both pre- and postsynaptically, and their stimulation leads to inhibition of adenylyl cyclase while activation of phospholipase C (PLC)[47,48]. These intracellular signaling cascades eventually lead to dephosphorylation of AMPA receptor subunits and depression of the synapse[49].

Stimulation of either NMDA or mGluR receptors alone is sufficient to induce LTD[50]. Furthermore, expression of LTD precedes LTP during brain development[51,52].

1.5 Cellular and molecular changes associated with LTP and LTD presynaptically

The major presynaptic mechanism associated with plastic changes at the synapse is alterations in the release rate of neurotransmitters[53]. Advancements in pharmacological tools made it somewhat convenient to validate postsynaptic components of synaptic plasticity in the 1980's. However, verifying the changes in neurotransmitter release rate associated with LTP had to wait until 1990's, until the developments in genetic, molecular biology, biochemical, proteomics and microscopic techniques. This is where SNARE proteins came in to the picture as well and helped explain the complexity of presynaptic terminals at central brain synapses.

Despite of the initial difficulties due to technological restraints, the classical studies of Josè Del Castillo and Bernard Katz in 1954[54] and Josèf Dudel and Stephen W. Kuffler in the 1960[55-57] provided compelling evidence of short-term facilitation and depression of the neurotransmitter release at the neuromuscular junction lasting hundreds of milliseconds. Facilitation represented probability (*p*) of neurotransmitter release. Since facilitation and

depression coexisted at the presynaptic locus, the direction whether *p* would slide towards facilitation or depression depended on in the initial *p*, with lower initial *p* favoring facilitation and vice-versa. In the 1990's researchers were finally able to experimentally show evidence of long-term changes in neurotransmitter release rate at hippocampal synapses associated with synaptic plasticity[53,58].

The current consensus is that synaptic plastic changes are associated with both pre- and postsynaptic modifications. Nevertheless, in view of the ever-increasing complexity of proteins being continuously implicated in, and associated with, these changes at both loci, we are still far from a complete understanding of synaptic plastic changes at the synapse, and how they account for the learning and memory encoding capability of the brain.

1.6 Hippocampus; an essential medial temporal lobe structure for the study of synaptic plasticity and memory

Bliss and Lømo chose hippocampus to record LTP in the first ever experimental recording of LTP. What motivated their choice at a time when no clear evidence was available of where the memories are stored in brain? They worked in Per Andersen's lab at the University of Oslo, and Per was an expert in studying hippocampus[59]. What was also, however, known at that time was the clinical case of patient Henry Molasin (H.M.) who had undergone bilateral temporal lobe resection, a surgical procedure which removed a major part of his temporal lobe, including the hippocampus in both hemispheres[60]. This procedure was performed in hope of a cure for his uncontrolled epileptic seizures, and it was successful in controlling his epilepsy, but left him with anterograde amnesia (unable to form new memories). Bilateral hippocampal lesions associated with the loss of episodic and semantic memories formation ability was reported in other patients as well[61]. This led experts to believe that hippocampus is crucial for the formation of new episodic (a memory with spatial and time coordinates) and semantic (memory of meanings/concepts) memories, because the older long-term memories, and general intellectual capabilities like language/words processing of H.M. were intact[62]. Some studies reported that only episodic memories rely on hippocampus, while semantic memories are partly dissociable to other brain regions as well[63]. Follow-up studies with patient H.M. also described that he was able to acquire some new semantic memories after years long training, perhaps with the help of some cortical brain areas[62]. Nevertheless, all the studies in human patients and animal models confer a crucial role to the hippocampus in the formation of explicit/declarative memories. While it might not be the site of permanent

storage of those memories, as evidence suggests parahippocampal cortices to hold intermediate memories, and neocortex as a final repository, hippocampal presence is certainly required for the normal formation and organization of declarative memories[64-66].

Bliss and Lømo's pioneering study and many other studies afterwards showed that hippocampus has a very organized laminar neuronal circuit, which is capable of undergoing plastic changes. The current view in the field of learning and memory research among neuroscientists is not much different from what it was more than 60 years ago, only with further additions and refinement of the hypothesized role of hippocampus. Human and animal studies with lesions of hippocampus, transgenic/gene-targeted knock-out/knock-in studies and pharmacological manipulations of the hippocampal circuitry affecting synaptic plasticity, have been shown to impair learning and formation of declarative memories[65,67-71]. Studies have also reported changes at the cellular physiology level as well, as learning via a behavioral task has been shown to induce LTP with AMPR delivery to the CA1 synapses in rodents[72]. The studies carried out in rodents, especially mice, are perhaps the most important in advancing our understanding of the cellular and molecular correlates of synaptic plasticity in hippocampus. Supplemented with behavioral paradigms, these studies provide a robust correlation between synaptic plasticity at hippocampal synapses and mechanisms underlying learning and declarative memory formation. In humans, however, it has not been easy to replicate all the animal studies due to the obvious scantiness of human brain material. However, some basic mechanistic aspects of synaptic plasticity induction and expression have been shown to be similar in rodents and in human brain tissue resected from epileptic and tumor patients, for example, NMDA receptor activation as a necessity for the induction of LTP[73,74]. But more importantly, clinical and post-mortem findings in human patients suffering from neurodegenerative diseases affecting learning and memories formation, for example, Alzheimer's disease, have established that hippocampus is a region of the brain severely affected[75]. Hippocampal atrophy resulting from synaptic degeneration/loss has been proposed to underlie the amnesic syndrome observed in those patients[76-78].

1.7 The hippocampal neuronal circuitry

In order to properly address the role of hippocampal synaptic plasticity in learning and memory formation, it is imperative to explain the hippocampal neuronal circuit organization with its inputs and outputs to the rest of the brain. Brain contains two hippocampi, one in each hemispheres in the temporal lobe, and each hippocampus resembles the shape of a

seahorse. Cross sectional anatomy of the hippocampus reveals a v-shaped wedge formation called the dentate gyrus (DG) which is composed of tightly packed layers of granule cells (GC). The GC receive their input via the perforant pathway, which is axons of glutamatergic neurons in the layer II/III of the entorhinal cortex (EC). GC extend their axons, mossy fibers (MF) and form synapses with pyramidal neurons in the CA3 region. The MF-CA3 synapses have been termed as one of the most powerful synapse in the brain and a salient feature of these synapses is that LTP can be induced there without the need for NMDA receptor activation. Axons from the pyramidal CA3 neurons transit through a relatively smaller sub-region, CA2, and form synapses with the pyramidal neurons in the CA1 area via the Schaffer collateral/associational/commissural fiber pathway. Schaffer collateral-CA1 synapses are the most extensively studied synapses in synaptic plasticity research. Axons of the pyramidal CA1 neurons project to layer V of the entorhinal cortex in the subiculum[79].

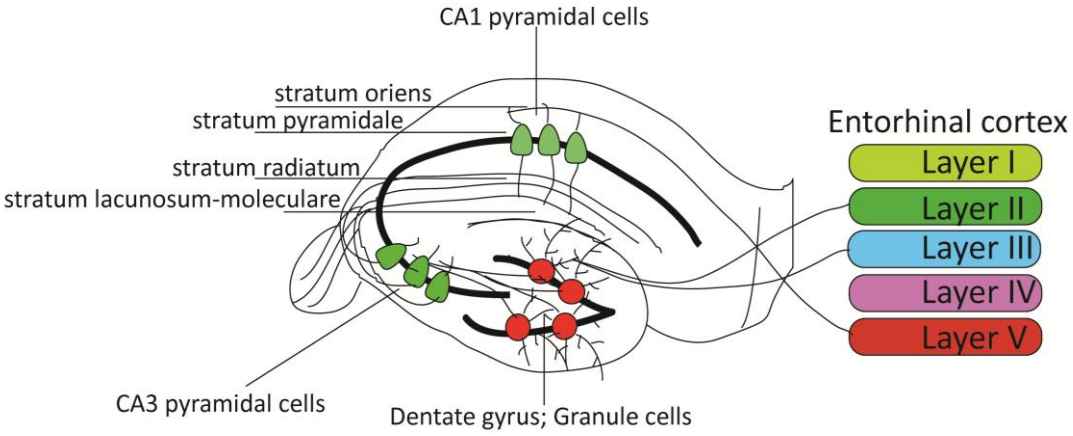

Figure 2: Neuronal circuit organization of the hippocampus with its major inputs-outputs.

1.8 Experimental approaches to record synaptic plasticity

The way to go to assess synaptic plasticity is recording electrical activity in the *ex vivo* acutely prepared brain slices. Different types of synaptic plasticity (STP, STD, LTP, LTD) can be recorded via this method, providing the convenience of combination with pharmacological manipulations and imaging modalities to carry out proof of concept studies. However, with convenience comes cynicism, especially when explaining a highly complicated phenomenon such as learning and formation of memories in brain's neuronal circuits. There are many questions, which cannot be answered by recording synaptic plasticity in *ex vivo* brain slices. Most prominent ones are, for example, the electrical activity recorded in brain slices when the brain is not in its natural native state, i.e. cannot be assumed to be exactly as electrical activity happening in brain *in vivo* during formation of memories. Critics of the LTP field also

argue that the HFS (100Hz) commonly used to induce LTP in brain slices in a laboratory setting is artificial. However, higher firing patterns of neurons have been shown *in vivo* as a substrate for activity dependent plasticity, for example place cells in the hippocampus[80,81], but there is no direct evidence of high frequency bursts associated with learning or formation of memories *in vivo*. A question which arises from the theory that 'higher activity of neurons leads to the synaptic connections becoming stronger' as a neural substrate for learning and memory formation, is the underlying assumption that the higher activity is triggered by a certain stimulus, which the brain deems essential to learn or remember. However, in reality, information is continuously fed to the brain, processed by streams of neuronal activity, but it is entirely unknown how it is decided what to learn and remember from all that information and what to discard. Last but not the least, as with all animal studies comes often the question of whether findings from *ex vivo/in vitro* experiments could also be present at the behavioral level. In addition, to what degree can animal data be extrapolated to humans, who evolutionarily have the most evolved brain and the highest cognitive abilities? That is why, for extrapolation and correlation of LTP data from brain slices to the behavioral level, a number of behavioral paradigms have been developed to test different learning behaviors primarily in rodents, but also in other species. Different behavioral tests rely on the neuronal activity of a specific area of the brain and serves to validate the *ex vivo/in vitro* findings and help screen pharmacological agents and study gene expression etc. in that brain region.

1.9 Behavioral testing in animals for assessing learning and memory formation

Only a short description of the most important behavioral tests for assessing learning and memory formation in animals (monkeys, rats and mice) is presented here, as over the years a large number of these tests have been developed. In the following, a few important and much used tests are described.

1.9.1 Delayed match to sample task

The prototype of this behavioral paradigm was developed in the 1950's, and pigeons were utilized as experimental models for assessing memory function[82]. The delayed match to sample task is a behavioral test, which can be applied to higher mammals (monkeys), rodents and humans as well, in different versions to assess retention of working/visual memory. For example, in one version of the test, a monkey will be presented an object on the screen for a short duration (few seconds), followed by a delay, two objects will be presented on the screen and the monkey is trained to make a selection for one object which will match the earlier

presented one. Correct matching is associated with a reward. *In vivo* electrical recordings in the brain during the course of experiment in monkeys have associated prefrontal cortex with working memory[83], but studies have also reported involvement of other brain regions like perirhinal cortex in rats [84].

1.9.2 Morris water maze

Morris water maze test developed by Richard Morris[85] and is the most commonly used behavioral test to assess hippocampal dependent spatial memory. Originally, it was developed for rats, but is commonly used now for testing mice as well, and different versions of the original paradigm exist. The basic principal is that rats or mice are subjected to swim in a large tank of opaque water with a hidden/visible platform, where they find the platform by co-incidence during training. In the actual test, the latency (time to the discovery of the platform) to find the platform is then monitored as a measure of learning and memory. This test also has evolved over the years and the test can be performed with/without spatial cues. Richard Morris was also the founder of two famous theories in the LTP field, i) 'synaptic tagging'[86] which states that transcriptional and translational factors are activated immediately after the potentiation of a synapse leading to early protein synthesis. Those proteins serves as tags and paves way for the late LTP. This has been proven with the application of protein synthesis inhibitors after induction of LTP with relatively weak tetanic stimulation. ii) The 'synaptic plasticity memory (SPM)' theory[87,88] states that activity-dependent changes in the synaptic efficacy in a specific brain region are both necessary and sufficient for the formation of a memory trace (also referred to as an 'engram') in that area of the brain.

1.9.3 Passive avoidance test

The passive avoidance test measures the retention time of a shock memory. The apparatus for the test consists of a brightly illuminated and a dark box with a through door from the light to the dark box. A rat or a mouse is placed in the bright box, and following their natural preference the animal transition to the dark box but here receives an electrical shock. The animal is removed from the dark box and administered a drug, and tested again to assess the retention of the shock memory. The behavioral tests used in the scope of this thesis are explained in the Material and methods section.

2 SNARE PROTEINS AND EXOCYTOSIS

2.1 Regulated membrane fusion

Regulated membrane fusion usually refers to protein-catalyzed lipid rearrangement of two adjacent membranes, the plasma and vesicle membranes, which eventually leads to the release of vesicle contents in a controlled targeted manner. This is a tightly regulated process with checkpoints and balances at every step by multiple regulatory molecules. The process is highly conserved, from yeast to higher vertebrates[89]. Regulated membrane fusion is not to be confused with lysis, as the permeability of the membrane to polar molecules is always intact. Regulated membrane fusion leads to the expulsion of proteins or transmitters in a targeted fashion[90-93]. The phenomenon of membrane fusion can broadly be divided into either 'constitutive' or 'regulated'. Intracellular membrane fusion of vesicles from endoplasmic reticulum (ER) fusing with Golgi apparatus are examples of a 'constitutive' membrane fusion event. This targeted release of proteins from ER to trans Golgi network and on to the cell surface with the help of signaling chaperone molecules, represents a mechanism which is present in almost all living cells, as cells are constantly synthesizing proteins[94,95]. However, the focus of this thesis is regulated membrane fusion, which leads to exocytosis of neurotransmitters from synaptic vesicles in neurons into the synapse. This is a property of excitable cells, including cells in the endocrine, neuroendocrine and nervous system, although the kinetics of hormones release significantly differ from neurotransmitters. In regulated exocytosis, proteins, peptides or small molecule transmitters are stored and packed in secretory vesicles and the release is triggered by a specific external stimuli leading to a rapid localized discharge of the vesicular contents[95,96]. The strict spatial and temporal control dynamics of regulated exocytosis in response to a well-defined triggering stimulus are what differentiates it from constitutive exocytosis. Membrane fusion (constitutive and regulated) lies at the core of vital processes of cell growth, hormone secretion and neurotransmission.

2.2 Regulated exocytosis, SNARE proteins and synaptic transmission

Both regulated and constitutive exocytosis are carried out with the help of Soluble *N*-ethylmaleimide sensitive factor Attachment protein REceptor (SNARE) complexes[97]. The SNARE family of proteins is made up by 35 members in *Homo sapiens* (human), 20 in *Drosophila melanogaster* (fly), 23 in *Caenorhabditis elegans* (worm) and 21 in *Saccharomyces cerevisiae* (yeast)[98,99] with many members having multiple isoforms. SNARE proteins are a large family of proteins with different members participating to form the trimeric core

complex in distinct (systems) excitable cells at different developmental time points. The core SNARE complex for regulated exocytosis of neurotransmitters is formed by three SNARE proteins, namely, Synaptosomsal associated protein of 25kDa (SNAP-25), syntaxin 1A and synaptobrevin 2 or vesicular associated membrane protein 2 (VAMP2)[97], hence, are also referred to as cognate neuronal SNAREs. The release of neurotransmitters at central synapses is a most tightly regulated process with controls exerted on a millisecond timescale. SNARE proteins constitute the functional machinery responsible for this highly regulated phenomenon at the presynaptic terminals. It is also worth noticing that different members of the SNARE family participate in the cell surface expression of the neurotransmitter receptors (constitutive release) at the postsynaptic terminals. For example, SNAP-23 has been shown to play a role in the exo- and endocytosis of NMDA receptors[100], similarly, another study has shown SNAP-25, syntaxin 4 and VAMP1 containing SNARE core complex to be responsible for constitutive exocytosis of NMDA receptors[101]. SNAP-25, SNAP-23, VAMP2 and syntaxin 1 have been shown to be responsible for $GABA_A$ and AMPA receptors exocytosis postsynaptically as well[102]. This makes SNARE proteins vital for the induction and expression of plastic changes at the central synapses as their involvement is inevitable. Changes associated with the plastic events at the synapse, for example, increase or decrease in the release probability of neurotransmitters or incorporation/removal of extra receptors have to be mediated through SNARE proteins, hence they hold high stakes in this important phenomenon.

2.3 NSF, αSNAPs and SNARE proteins

The now diversified field of membrane fusion was driven forward by the pioneering work of James Rothman, Randy Schekman and Tomas Südhof in the late 1980's. Schekman's work explained impairments in intracellular protein trafficking pathways mediated by *SEC* genes in *Saccharomyces cerevisiae* (yeast)[103]. Rothman and colleagues isolated a 76 kDa homo-oligomer called; *N*-ethylmaleimide (NEM) sensitive factor (NSF) from virus infected CHO cells[104,105]. NSF was quickly recognized as a crucial component of the Golgi transport and intracellular fusion system and it required additional cytoplasmic factors to function called soluble NSF attachment proteins (SNAPs). Inactivated Golgi transport by NEM could be rescued by the addition of the NSF and α-SNAPs[104]. Südhof's work focused on neurotransmitter exocytosis and identified synaptotagmin (previously known as p65)[106] and Munc-18 (mammalian homologue of unc-18)[107] as crucial components of the regulated exocytosis of neurotransmitters. All three scientists shared the Nobel Prize in 2013.

The experiment that led to the three SNARE proteins implicated in regulated neurotransmitter exocytosis was sort of a fishing expedition. Researchers moved from intracellular constitutive exocytosis and wondered what could bind to NSF from bovine brain lysates. An affinity chromatography assay, which utilized the principle of natural binding of NSF protein to its receptors from bovine brain, demonstrated three proteins, SNAP-25, syntaxin B and VAMP2, to be binding targets of NSF[108]. These proteins were termed as soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). Following the discovery of SNARE proteins, a 'SNARE hypothesis' was put forward stating that SNARE proteins can interact to form a core complex which is necessary for regulated exocytosis[97]. This hypothesis was based on the findings that the three SNARE proteins could interact to form a complex in the absence of NSF and αSNAPs and the core complex can interact with synaptotagmin. The proof of concept that SNARE proteins are indeed the mediators of membrane fusion leading to exocytosis came from another study, in which the researchers incorporated purified recombinant SNAREs in to liposomes (vesicles with a 50nm diameter)[109]. The design of the assay was already published[110]; a donor liposome containing fluorescent tagged lipids, while the acceptor was non-fluorescent. Mixture of liposome contents up on fusion, led to dequenching of the fluorescent probe, confirming the lipid rearrangement and membranes mixing. This study also showed, that these three SNARE proteins could form the minimal machinery required for fusion and exocytosis. In parallel studies, researchers also showed, that SNARE proteins are substrates for the proteolytic activity of Clostridium (Botulinum neurotoxins, BoNT)[111] and Tetanus endoproteolytic neurotoxins[112]. BoNT types A and E cleave SNAP-25, type C cleaves syntaxin and SNAP-25 and type B, D, G and F act on VAMP[113,114]. Of all these neurotoxins, the most extensively studied are the effects of BoNT type A and E on SNAP-25 and how they impair the evoked neurotransmitter release.

2.4 Classification of SNARE proteins

As mentioned earlier, the SNARE family of proteins is quite large with more than 30 members in humans, but the focus here is only on the three SNARE proteins involved in regulated neurotransmitter exocytosis. The initial finding that the three SNARE proteins responsible for neurotransmitter exocytosis are localized in the target plasma or vesicular membranes led to their classification accordingly. SNAP-25 and syntaxin 1A were found to be attached to the target plasma membrane, and were termed t-SNAREs, while VAMP2 was found to be

attached in the vesicular membrane and was termed v-SNARE [108]. This classification could still be used for describing SNAREs' role in regulated exocytosis in neurons and synaptic transmission. However, given the universality of SNARE proteins in other forms of exocytosis such as intracellular constitutive exocytosis from endoplasmic reticulum to Golgi network, which can be bi-directional (anterograde or retrograde) and may involve differential pairing of SNAREs, the 't' and 'v' SNARE classification can at times be confusing. Knowledge of how SNARE proteins interact to form a highly stable core complex, along with the resolution of the crystal structure of the SNARE core complex, revealed a highly conserved sequence of ionic residues in the middle of the SNARE core complex[115]. This ionic residue layer consisted of three glutamine (Q) and one arginine (R) and was embedded deeply within the 4 parallel helical bundles of leucine-zipper-like layers. Based on this finding, SNARE proteins were re-classified as Q (glutamine) and R (arginine) SNAREs[116]. It was postulated that for a core complex to be highly stable and fusion competent, it has to have 3 Q and 1 R SNARE. Again, in the context of SNAREs mediated neurotransmitter exocytosis at central synapses, R-SNAREs correspond to v-SNARE while Q-SNAREs correspond to t-SNAREs, hence, VAMP2 provides one R-residue, and syntaxin 1A provides one Q-residue while SNAP-25 provides two Q-residues[117].

2.5 Formation of SNARE core complexes

A SNARE protein is characterized by the presence of eight heptad repeats of hydrophobic residues, an evolutionary conserved stretch of 60-70 aminoacids called the 'SNARE motif'[118]. For syntaxin 1A and VAMP2, this motif is located next to the single transmembrane domain (C-terminus), serving to localize the cytoplasmically soluble protein. However, SNAP-25 does not possess a trans-membrane domain and possess two SNARE motifs. SNAP-25 is anchored via post-translational palmitoylation of the cysteine-rich region in a linker region between the C- and N-terminus amphipathic helices [119,120]. The favored hypothesis is that post-translational palmitoylation helps SNAP-25 localize to the fusion site but it is also worth noticing that SNAP-25 forms heterodimers with syntaxin 1A[121].

The SNARE core complex is formed when four SNARE motifs assemble in parallel four α-helices coiled-coiled bundle[122]. The core complex is strengthened via a process called 'zippering'[123]. Of the four SNARE motifs in the core complex, two are supplied by SNAP-25 (Qb and Qc motifs) and one each by syntaxin 1A (Qa motif) and VAMP2 (R motif)[91,92,97,124,125]. Formation of these helical bundles from members located on opposing membranes is

achieved through the process of tethering[126], (in which the fusing membranes are brought close to each other). Tethering requires ancillary proteins, for example, Rab proteins present on synaptic vesicles and active zone RIM proteins[127]. Once the membranes are tethered, VAMP2 is in a parallel proximity with heterodimers of syntaxin 1A and SNAP-25. At this stage of exocytosis, the core complex is considered to be loosely bound, as it is susceptible to the actions of clostridial neurotoxins[113,114]. A tight and highly stable core complex forms afterwards in a zipper-like fashion between VAMP2 and syntaxin 1A beginning from the N-terminus of VAMP2 and traveling towards its C-terminus[128]. In this tight trans-configuration-mode, the core complex is resistant to the action of the above mentioned neurotoxins[129]. Rises in $[Ca^{2+}]_i$ via opening of the voltage-gated Ca^{2+} channels (VGCCs) is the initiator of this trans-complex formation[130]. Formation of the tight SNARE core complex generates enough energy, which overcomes the energy barrier for lipids rearrangements and fuse the two membranes. After the fusion reaction, the core complex rests in a cis-configuration. At this point, disassembly of the core complex is initiated by the action of ATP-dependent NSF, which alone cannot dismantle the core complex but requires co-factors, as mentioned before, soluble NSF attachment proteins (αSNAPs) (different proteins from SNAP-25). Together with SNAPs and the energy derived from ATP, NSF dismantles the in-active SNARE core complex and ensures an uninterrupted supply of the SNARE proteins and a steady state neurotransmitter release[131].

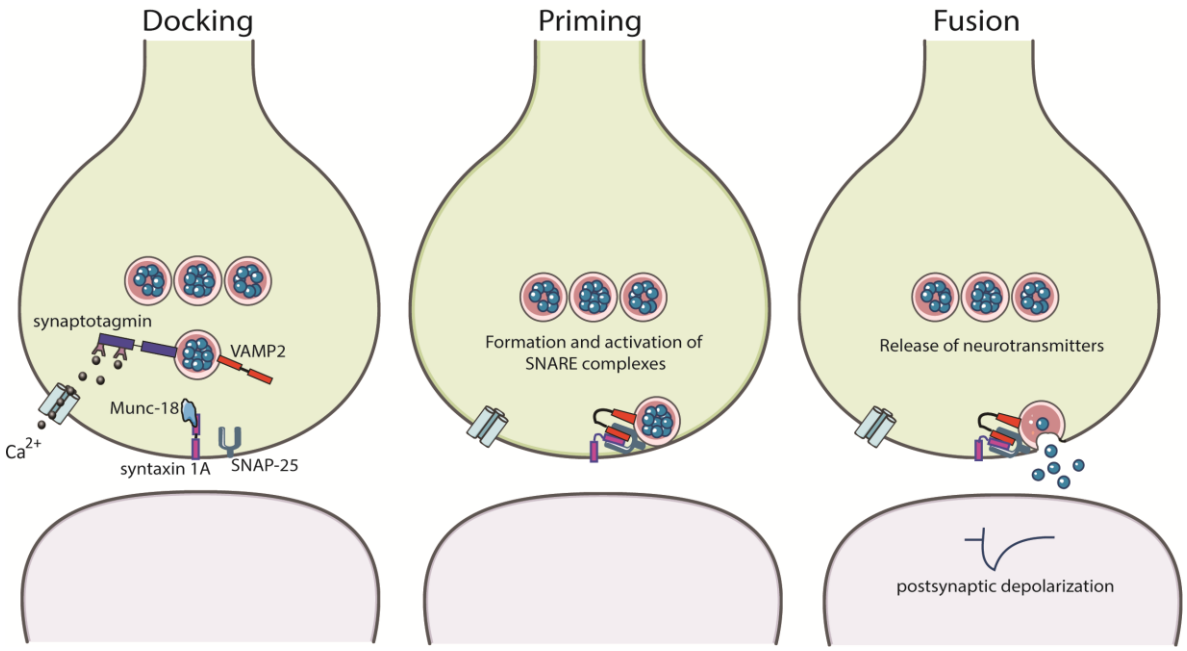

Figure 3: Sequential steps in regulated neurotransmitter exocytosis.

2.6 Ca^{2+}; a trigger for neurotransmitter exocytosis

Rises in $[Ca^{2+}]_i$ levels is sensed by synaptotagmin I, a synaptic vesicle protein with two cytoplasmic Ca^{2+} binding domains called C2A (can bind 3 Ca^{2+} ions) and C2B (can bind 2 Ca^{2+} ions), see review[132]. Synaptotagmin functions as a clamp but when intracellular Ca^{2+} concentration increases, the break is released. The resting intracellular Ca^{2+} level in a neuron typically ranges between 50-100 nM, while extracellular concentrations are approximately 2mM under normal conditions. An increase to 1-2 µM can initiate vesicle fusion in presynaptic terminals. During an action potential, levels rapidly increase to as high as 10 µM because of the opening of the VGCCs, causing depletion of the readily-releasable pools of neurotransmitter containing vesicles (RRP) [133]. Ca^{2+} uncaging experiments at the brainstem auditory giant synapse of the Calyx of Held, has provided insight on how a rise in Ca^{2+} level in the pre-synaptic terminal relates to the regulated release of neurotransmitter. Elevated Ca^{2+} levels triggers release in <400 µsec, which means that Ca^{2+} is sensed very rapidly by a sensor and a fusion pore is formed in quick succession, implying that once triggered, it is an energetically-favored process. The rapid phase is followed by a relatively slower phase, which is due to the residual Ca^{2+} levels when it is being buffered[133-135].

The number and size of the RRP of vesicles vary between synapses. A synaptic vesicle is a small organelle of approximately 40 nm diameter[136], which expresses transport and trafficking proteins on its surface to be able to undergo exo/endocytosis of neurotransmitters. The number of synaptic vesicles in the RRP at the presynaptic locality ranges from 200-400[137,138]. The number of vesicles in the RRP can be altered by a number of factors, for example, brain-derived neurotrophic factor [139], SNAP-25 isoforms[140] and many more.

2.7 Ancillary SNARE interacting proteins

Co-immunoprecipitation and pull-down assays have shown synaptic SNARE proteins to interact with more than 100 proteins[91]. Proteins that are pivotal in regulating the entire process of exocytosis at presynaptic terminals can be broadly grouped as, a) the core exocytotic proteins, b) ancillary/auxiliary proteins which are critical for the formation of SNARE core complex and docking and priming of neurotransmitter containing secretory vesicles, their recycling as well, c) ion channels, voltage dependent Ca^{2+}, Na^{+} and K^{+} channels, d) calcium sensing proteins, can be grouped under accessory proteins or separately and e) presynaptic inhibitory G protein-coupled receptors (GPCRs). A short description of the important proteins from these classes is presented here.

SM proteins (Sec1/Munc-18) bind to syntaxin 1A in its closed conformation and prevent its participation in SNARE core complex formation. There is also evidence of SM proteins binding to open conformation of syntaxin 1A as well, suggesting a more complicated regulatory role for them[141-144]. Interestingly, deletion of VAMP2 abolished 90% of Ca^{2+} triggered exocytosis[145] while knocking out Munc18-1 completely abolished it[146]. Munc18-1 is critical for the formation of trans-SNARE parallel complexes, which bring the fusing membranes close to each other. There is evidence that Munc-18 selectively activates/mobilizes cognate neuronal SNAREs for initiating exocytosis, and at the same time, suppressing/preventing other ubiquitous SNAREs from participation in this process[147]. There is also evidence of Munc-18 promoting nucleation and zippering of the SNARE core complex[148].

Synaptotagmins: Ca^{2+} is sensed by a synaptic vesicle proteins called synaptotagmins, synaptotagmin I is the most abundant isoform [132,149]. Synaptotagmin is essential for the fast Ca^{2+} triggered synchronous phase of exocytosis but not for the slow phase[150,151]. Deletion of synaptotagmin in mice produced a lethal phenotype[152]. After binding Ca^{2+}, synaptotagmin I interacts with syntaxin 1A and SNAP-25 to promote rapid exocytosis of neurotransmitters.

Complexins: They are small helical cytoplasmic proteins, which bind to the surface of both partially and completely assembled SNARE complexes[153]. They are dislodged from SNARE complex by synaptotagmin as Ca^{2+} bound synaptotagmin 1 competes with complexins for binding to SNARE complexes. Complexins are hypothesized to have a role in stabilizing SNARE core complexes before Ca^{2+} triggered fast exocytosis and regulating SNARE functions[154]. Complexins have also been termed 'fusion clamps' since they prevent the SNARE core complex from initiating fusion before being disrupted by Ca^{2+}-bound synaptotagmin[155].

Rab Proteins: During the process of fusion, GTP bound Rab proteins acts as anchors on membrane surfaces for the target effector proteins. Rab proteins cause tethering (process of bringing membranes closer to each other) with the help of their effector proteins and are critical for the induction of plastic changes at central synapses[156-159]. Rab-effector complexes also enrich the environment of the tethered membranes with SNARE proteins[91,160]. There are multiple isoforms of Rab proteins and there is evidence of functional redundancy between these isoforms as KO mice models of individual isoforms of Rab proteins did not affect survival but knocking out multiple Rab proteins proved lethal[161].

2.8 Presynaptic G protein-coupled receptors (GPCRs)

Activation of cell surface G protein-coupled receptors (GPCRs) by a ligand leads to the dissociation of intracellularly coupled heavy heterotrimeric G protein in to Gα monomer and GβϒY dimer, see review[162]. Both these subunits are activated after dissociation and are known to perform a vast array of functions within the cell in the capacity of secondary messengers. Initial research after the discovery of heterotrimeric G proteins mostly reported the roles of Gα subunit but now, more roles for the GβϒY subunit are emerging[163-165]. The GβϒY subunit, is a dimer but, can be considered as a monomer because of the strong association between the β and ϒ subunits, and naturally GβϒY together is physiologically relevant rather than the units alone[166,167]. GβϒY subunits have been shown to negatively modulate neuronal excitability by i) interacting directly with presynaptic voltage-gated Ca^{2+} channels and prevent Ca^{2+} entry in to the cell[168,169], ii) postsynaptic GIRK channels[170,171]and iii) the inhibitory role of GβϒY subunits is also exerted downstream of ion channels and directly on to the exocytotic SNARE fusogenic machinery[172]. Direct evidence of GβϒY mediated reduction in glutamate release comes from the study of 5HT mediated blockade of glutamate release[173]. GβϒY interacts with the C-terminal region of SNAP-25[174], and this interaction has been shown to negatively affect the Ca^{2+} dependent exocytosis of hormones and neurotransmitters, presynaptic inhibition[172,175]. BoNT/A and alanine mutagenesis studies have shown that GβϒY dimers binds to a region on the C-terminus of SNAP-25 (residues 193-206)[176]. The physiological relevance of the role of GβϒY comes from the finding that it competes with synaptotagmin I for binding to ternary SNARE complexes and is negatively associated to exocytosis. In conditions of high Ca^{2+} concentrations, synaptotagmin I wins this competition and promotes exocytosis[177,178]. Prof. Heidi Hamm and colleagues have developed a mouse model (SNAP-25Δ3) in which they have mutated residues on the extreme of the C-terminus of SNAP-25. This mutation reduces the ability of GβϒY to bind to SNAP-25 by two-folds, hence reducing the inhibitory actions of $G_{i/o}$-coupled GPCRs on exocytosis, while the ability of SNAP-25 to bind to synaptotagmin I is still intact [179]. Our group has shown that SNAP-25a and SNAP-25b interact differently with GβϒY subunits[180]. Prof. Patric Stanton and colleagues have shown that the interaction of GβϒY with the C-terminus of SNAP-25 is necessary for the induction of presynaptic long term depression (LTD) of vesicular release, but not long term potentiation[181].

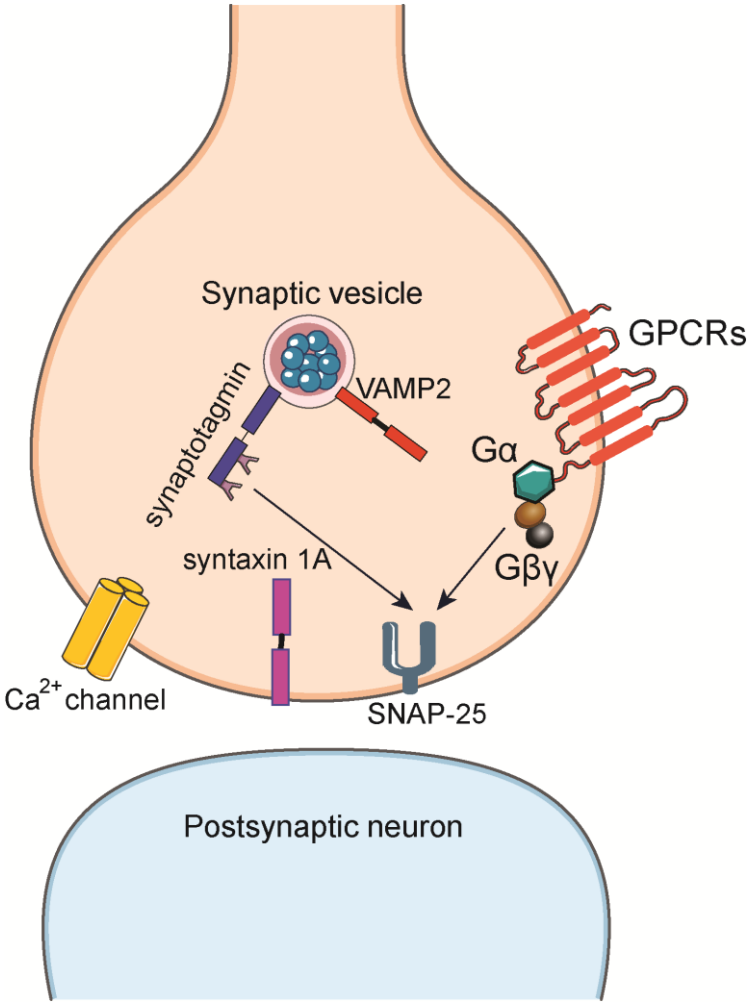

Figure 4: Schematic representation of inhibitory presynaptic GPCRs interaction with SNAP-25.

2.9 Alternative splicing of the *Snap25* gene

The gene encoding for SNAP-25 is a single copy gene comprised of 9 exons spaced by large introns[182]. Characterization of the *Snap25* gene revealed two closely related exon 5 sequences spaced with a small intron. This led to the buildup of knowledge about obligate alternative splicing and the existence of two closely related isoforms in SNAP-25 ('a' and 'b'). The two isoforms of SNAP-25 differ by 9 out of 39 residues encoded by two different exon 5[182,183]. These differences are localized at the end of the N-terminal amphipathic helix, which continues into a linker region between the N- and C-terminal amphipathic helices. Here, four cysteine residues are clustered and are target sites for post-translational palmitoylation[119]. SNAP-25a and SNAP-25b are developmentally and neuroanatomically regulated[184], SNAP-25a is expressed at embryonic earlier stages than SNAP-25b and throughout life in selected cellular structures. In mouse brain, a developmental switch from SNAP-25a to SNAP-25b occurs, and after the second postnatal week, levels of SNAP-25b mRNA increase, ultimately leading to the 'b' isoform being the abundant (>90%) isoform in adult mouse brain[185,186].

SNARE complexes containing SNAP-25b have been shown to be more stable than those containing SNAP-25a[186]. Expression of exogenous SNAP-25b in SNAP-25 null mutant fetal chromaffin cells has been shown to prime a larger group of pooled vesicles than SNAP-25a, supporting the notion of functional differences between both the isoforms and that the 'b' isoform is possibly capable of mediating fusion from a larger group of primed vesicles[140,187].

Dr. Christina Bark has engineered a gene targeted mouse mutant utilizing a minimally disruptive approach to the organization of the *Snap25* gene, allowing the dissection of the functional differences between the two isoforms. The downstream sequence of exon 5 encoding for the 'b' isoform has been replaced with an additional 'a' isoform encoding sequence, hence allowing the alternative splicing switch to function, but results in a global production of only SNAP-25a[188].

2.10 Significance of SNAP-25 and its isoforms in diseases

Polymorphisms in the *Snap25* gene have been associated with neurodegenerative and psychiatric disorders including ADHD, autism, bipolar disorders, epilepsy and schizophrenia[189-191]. The coloboma mouse model with deletion of *Snap25* gene sequences, exhibited hyperactivity (ADHD like behavior)[192]. Increased interaction between SNAP-25 and other SNARE/ancillary proteins have been proposed as an underlying mechanism for synaptic dysfunction in schizophrenic patients[193-195].

SNAP-25 is not only relevant for synaptic transmission, plasticity and neurological diseases but also important for peripheral metabolic functions. For example, neuroendocrine and endocrine hormones secretion is carried out through SNARE mediated membrane fusion as well[196]. Our group has shown that replacing SNAP-25b with SNAP-25a in mouse results in metabolic abnormalities like hyperglycemia, liver steatosis, adipocyte hypertrophy, abnormal weight gain/obesity and hyperinsulinimeia[197,198].

3 AIMS OF THIS THESIS

The general aim of this thesis was to evaluate if the two isoforms of SNAP-25 differ in their ability to confer plastic changes to the hippocampal Schaffer collateral-CA1 synapses. The specific aims of this thesis were the following.

1. Differentiate the role of SNAP-25a from SNAP-25b in synaptic tranmission, induction of LTP and determine the possible consequences for learning and memory formation, utilizing a gene-targeted mouse model expressing only SNAP-25a.

2. Study the developmental time course of SNAP-25 isoforms regulation of both LTP and LTD at hippocampal synapses, and assess the possible consequnces for learning and memory formation.

3. How do mutations in the extreme C-terminus of SNAP-25 affect induction and expression of LTP?

4. Evaluate if the metabolic phenotype conferred by the lack of SNAP-25b and Western diet affects cognitive function as assessed by various behavioral paradigms, and correlate those changes with SNARE proteins expresssion levels in different brain regions.

4 MATERIAL AND METHODS

4.1 Animals

The complete protocol for generating genetargeted SNAP-25b-deficient mice is described in Johansson. et al. (2008)[186]. An animal colony was established and the breeding and experimental protocols were approved by Stockholm Northern Animal Experiments Ethics Board (Ethical Permit # N33/14), and performed according to the standards and guidelines in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. An animal colony of SNAP-25b-deficient mice (breeding pairs supplied by Dr. Christina Bark), and SNAP-25Δ3 mice (breeding pairs supplied by Dr. Heidi Hamm, Vanderbilt University) was also established at New York Medical College, Valhalla, New York, U.S.A. Breeding and experimental protocols in US were also approved by the Institutional Animal Care and Use Committee (IACUC Ethical Permit # 11-12-0315) of New York Medical College Valhalla, New York, U.S.A. Experiments at New York Medical College were performed in accordance with Association for Assessment and Accreditation of Laboratory Animal Care, Intl., (AAALAC) standards and guidelines. Animals were provided access to food and water *ad libitum*, and euthanized under deep isoflurane anesthesia, unless otherwise stated.

4.2 Diet

In paper I, II and manuscript I, mice were fed standard chow, control diet (CD) *ad libitum.* In manuscript II, mice were divided in to four different experimental groups depending on the genotype and diet. These groups were, wild type (WT) on CD, SNAP-25b-deficient (MT) fed CD, WT on Western diet (WD) and MT on WD, (males and females in separate cohorts). WD (high-fat/high-sucrose) was purchased from Research Diets Inc®(New Brunswick, NJ, USA). WD intervention was started at the age of 5 weeks, and continued for 7 weeks and mice were euthanized for experiments when they turned 12 weeks of age.

4.3 Brain slice electrophysiology experiments

For electrophysiological recordings in brain slices, mice were deeply anesthetized with the help of isoflurane, decapitated and the brains were quickly dissected out. The cerebellar part of the hindbrain and prefrontal cortex were removed, the brain was hemisected through the mid-sagittal plane and immersed in a chilled high Mg^{2+}, sucrose-based cutting solution containing 87mM NaCl, 25mM $NaHCO_3$, 25mM glucose, 75mM sucrose, 2.5mM KCl, 1.25mM

NaH_2PO_4, 0.5mM $CaCl_2$ and 7mM $MgCl_2$ (continuously equilibrated with 95% O_2-5% CO_2 gas mixture). High Mg^{2+} prevents excitotoxicity. Individual brain lobes were fixed to a stage, with the frontal part of the brain touching the stage, with cyanoacrylate adhesive, and 350-400 µm thick coronal sections were cut with a Leica model VT 1200S vibratome®(Leica biosystems®). Slices were incubated in an interface holding chamber containing the same cutting solution for approximately 20 min at 32°C, and then transferred to normal artificial cerebrospinal (aCSF) fluid containing: 126mM NaCl, 3mM KCl, 1.25mM NaH_2PO_4, 1.5mM $MgCl_2$, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, 10mM glucose and continuously bubbled with 95%O_2-5%CO_2. Slices were allowed to recover for 1h at room temperature in aCSF before transfer to an interface recording chamber perfused continuously with aCSF at 3ml/min.

4.4 Inducing long-term potentiation of synaptic strengh (LTP)

For recording of activity-dependent LTP, slices were transferred to an interface recording chamber and continuously perfused by oxygenated aCSF at 3ml/min, and the chamber temperature maintained at 32 ±0.5^{o}C. Field excitatory postsynaptic potentials (fEPSPs) were recorded at Schafer collateral–CA1 synapses in the hippocampus. A borosilicate thin walled glass recording electrode filled with aCSF (1–2MΩ) was placed in field CA1 stratum radiatum. Half-maximal fEPSPs were evoked using a bipolar tungsten stimulating electrode (FHC Co. Bowdoin, ME, USA.) placed near the recording electrode. fEPSP slopes were confirmed to be stable to within ±10% for at least 10 min before commencing an experiment. Single shock evoked fEPSPs were acquired every 30 seconds. LTP was induced by application of high frequency theta burst stimulation (HFS or TBS), and LTP measured as the ratio of mean slope at indicated time intervals post-TBS normalized to the pre-TBS baseline slope. TBS stimulation consisted of 2 stimulus trains 3 min apart and each train consisting of 10 stimulus epochs delivered at 5 Hz (200 ms apart), and each epoch consisting of 5 pulses at 100 Hz. Constant current was injected through an ISO-flex isolator, AMPI® triggered by Master-8® pulse generator. Signals were amplified with an A-M 1700 differential AC Amplifier (A-M Systems®), and digitized by an A/D board (National Instruments®) controlled by Sciworks software (Datawave Technologies®).

4.5 Inducing long-term depression of synaptic strength (LTD)

The experimental setup for recording of LTD in brain slices was the same as for recording LTP, except instead of HFS, prolonged low frequency stimulation (LFS) was used. LFS for the induction of LTD consisted of a single train of 2Hz stimulation for a period of 10 min (1200 ×

150 μs duration dc square pulses). mGluRII LTD was induced by 5 min bath application of 25μM DCG-IV (Tocris, Bio-Techne corporation, MN). NMDA LTD was induced by 3 min bath application of 20μM NMDA (Sigma-Aldrich).

4.6 FM1-43 imaging

Fluorescence was visualized using a customized two-photon laser-scanning Olympus BX61WI microscope with a 60x/0.90W water immersion infrared objective lens and an Olympus multispectral confocal laser scan unit. The light source was a Mai-Tai™ laser (Solid-State Laser Co., Mountain View, CA), tuned to 820 nm for exciting FM1-43. Epifluorescence was detected with photomultiplier tubes of the confocal laser scan head with pinhole maximally opened and emission spectral window optimized for signal over background. In the transfluorescent pathway, a 565 nm dichroic mirror was used to separate green and red fluorescence to eliminate transmitted or reflected excitation light (Chroma Technology, Rockingham, VT). After confirming the presence of Schaffer collateral-evoked fEPSPs >1 mV in amplitude in CA1 *stratum radiatum*, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was bath-applied throughout the rest of the experiment to prevent synaptically-driven action potentials in the pyramidal neurons and prevent the accelerated dye release. Presynaptic boutons were loaded by bath-applying 5 μM FM1-43 (Molecular Probes) in hypertonic ACSF supplemented with sucrose to 800 mOsm for 25 sec to selectively load the RRP, then returned to normal ACSF. Stimulus-induced destaining was measured after 30 min perfusion with dye-free ACSF, by 0.1 Hz bipolar stimuli (150 μs DC square pulses).

4.7 Western blotting

For Western blotting, animals were euthanized through decapitation; brains quickly removed and cooled down with ice cold 1×PBS. Cerebellum and prefrontal cortex were removed, then the brains were hemisected, both hippocampi extracted and the individual brain regions were flash frozen immediately at -80 °C in liquid nitrogen. Tissues were stored at -80°C until further processing. For making lysates, tissues were homogenized in ice-cold buffer containing 100 mM NaCl, 20mM Hepes, 1mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, 5mM DTT, 20% (vol/vol) glycerol, 1 tablet/10ml (complete mini® protease inhibitor; Roche Diagnostics GmbH) and 1 tablet/10ml (complete mini® phosphatase inhibitor; Roche Diagnostics GmbH). Tubes containing homogenates were exposed to a thermal shock at -80 °C in liquid nitrogen and thawed to 37 °C. This freezing-thawing cycle was repeated three consecutive times and afterwards samples were centrifuged at 10,000×g for 20 min and the supernatant was

collected. Protein levels in the supernatant of individual sample homogenates were measured through the Bradford standard curve method, and volumes were adjusted in Laemmli buffer [50mM Tris (pH 6.8), 10% (vol/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) beta-mercaptoethanol, and 2mg/mL bromophenol blue] to 2µg/µL of protein concentration. Equivalent amounts of proteins (20 µg) were loaded and ran on 4–12% Bis-Tris mini gels (NuPAGE®, Life Technologies) in MES buffer (Novex®, Life Technologies) at constant voltage (200 V) for 35 min and transferred to Nitrocellulose membranes using iBlot®(Thermo-Fisher scientific) 7 min dry transfer apparatus. Membranes were blocked with 5% (wt/vol) skim milk powder (Sigma-Aldrich) in 1X PBS solution, washed with washing solution (0.1% skim milk powder, 0.05% vol/vol Tween-20® in 0.5X PBS solution) and the membranes were carefully cut at the respective molecular weight of the protein of interest. Strips of the membrane were incubated overnight at 4 °C with primary antibodies directed against SNAP-25A (Rabbit Polyclonal: dilution 1:1000; Synaptic Systems), SNAP-25B (Rabbit Polyclonal: dilution 1:1000; Synaptic Systems) total SNAP-25 (Rabbit Polyclonal: dilution 1:20,000; Synaptic Systems), Syntaxin 1A (Mouse monoclonal HPC-1: dilution 1:20,000; Sigma-Aldrich), VAMP2 (Mouse monoclonal: dilution 1:20,000; Synaptic Systems), SNAP-47 (Rabbit Polyclonal: dilution 1:5000; Synaptic Systems) and Beta-actin (Mouse monoclonal: dilution 1:8000; Sigma-Aldrich). After incubation with secondary antibodies for one hour at RT, (anti-mouse or anti-rabbit IgG-peroxidase complexes; GE Healthcare), blots were incubated in commercial enhanced chemiluminescence reagents (ECL-Prime, GE Healthcare) and membranes exposed to a chemiluminescent CCD camera (Las-1000 Plus; Fujifilm, Tokyo, Japan). Densitometry quantification on the 16bit.tiff images were performed using ImageJ software (National Institutes of Health, Bethesda, MD).

4.8 Behavioral tests

4.8.1 Active place avoidance learning task

The active place avoidance task used was described previously by Burghardt et al.[199]. In this paradigm, mice are placed on a circular rotating platform that continuously turns clockwise at a speed of 1 rpm. Over several days and multiple trials (Fig. 3a), mice learn to identify the 60° shock zone guided by spatial markers on the walls surrounding the apparatus. Entrance into the shock zone triggered a brief constant foot-shock (500ms, 60Hz, 0.2mA) with an inter-shock interval of 1.5s that would cease upon leaving the shock zone. The middle point of the animal was used as the reference point to determine the position of a mouse and recorded

using behavioral analysis software (AnyMaze, Stoelting Co., Inc). Using the same software, the number of shock-zone entries was measured, where a decrease in shock-zone entries indicates learning. During the initial pre-training trial (10 min) when the shock was turned off, mice were allowed to habituate to the apparatus and showed no preference for any area of the platform. Subsequently, the shock was turned on and the mice completed 3 training sessions (10 min each) per day for 3 days, followed by an extinction trial (10 min) on the next day. Then the shock was turned off and the animals were allowed to ambulate freely into the zone previously associated with the shock. After extinction, a conflict variant task was performed in order to test cognitive flexibility. The shock zone was moved 180° from where the original shock zone was placed, and 3 conflict-training sessions (10 min each) were conducted per day for 2 days. Mice had to avoid the new shock zone which requires cognitive flexibility. This is represented in the task by the simultaneous suppression of the learned condition response of avoiding the original shock zone and learning the new association of a foot-shock with a different zone.

4.8.2 Elevated plus maze test

The elevated plus maze (EPM) is used as a general indicator of anxiety. The apparatus consists of two open arms (50 x 10 cm) and two closed arms (50 x 10 x 40 cm), connected by a center platform (10 x 10 cm) made of opaque dark grey plexiglass (Stoelting Inc., USA). The arms of the EPM are elevated 50 cm above the floor. Animals (n = 12-17) were placed in the center platform of the EPM, facing an open arm and allowed to explore the maze for 5 min. The middle point of the animal was used as the reference point to determine the position of a mouse and recorded using behavioral analysis software (AnyMaze, Stoelting Co., Inc). Percent of time spent in the open arms was calculated, where a decrease in percent of time spent in the open arms indicates an anxiety phenotype.

4.8.3 Novel object location test

The novel object location test is a hippocampal dependent behavioral task[200,201] designed for rodents. This task utilizes the rodent's innate curiosity to explore an object more when its relative position is changed versus the previous position, as the rodent initially learnt it. We employed this test for evaluating hippocampal dependent learning in WT and SNAP-25b-deficient mice both on control diet and after 7 weeks of Western diet intervention. Testing platform consisted of an open-field box made of wood (25 cm long x 25 cm wide x 25 cm

high). Objects utilized during the test as stimuli for the mice to explore and learn were identical Lego pieces (Lego, Slough, UK). There were no external cues for the mice.

The test consisted of three sessions/phases: (i) Exploration session: During this phase, animals were allowed to explore the arena freely for 10 min and got acquainted with the objects. Both the objects were placed 5 cm from top left and right corners. The position of one object was fixed, which would serve as the 'familiar' object, while the position of the other object was changed during the test 'novel' object. Mice were always introduced in the box with their backs towards the objects and were returned to their cage at the end of each session. (ii) Two 'retention sessions' were performed 1- and 24-hours after the exploration session. In this case, mice were allowed to re-explore the arena for 5 min. During the first retention session (1 h after exploration session), the familiar object remained in its same position as during the 'exploration session', but the novel object was presented 5 cm from the bottom right corner. (iii) During the second retention session (24h after the first retention session), animals were allowed to explore the arena for a 5 min period, but the novel object was now presented 5 cm from the bottom left corner. Activity of the mice was recorded with a cell phone video camera, and a discrimination ratio was obtained by dividing the time spent with the novel object divided by the total time spent with the novel and familiar objects.

4.8.4 Forced swim test

The forced swim test is a commonly used behavioral test to evaluate the anti-depressive effects of anti-depressant medications. We employed this test to assess, if lack of SNAP-25b with or without Western diet produces depressive like symptoms in mice. During this behavioral test, each mouse was briefly placed in an open glass cylinder (25 cm high, 14 cm wide) containing water (20 cm depth) maintained at 25 ±2^{o}C. Mice were forced to swim for 6 min and video recorded with cell phone camera. Latency (time to the first bout of immobility) and the total immobility times were recorded and analyzed.

4.8.5 Rotarod test

The rotarod test is a frequently used behavioral test for assessing motor coordination/learning and balance. We employed this test to assess the motor coordination in WT and SNAP-25b-deficient mice fed control and Western diet. The rotarod apparatus (Ugo Basile, Varese, Italy) consisted of a rotating rod (3 cm in diameter) divided into 5 running lanes allowing up to 5 mice to be tested simultaneously. The test was conducted in two phases, pre-

test and actual test. In the pre-test phase, mice were placed on the cylinder rotating at a fixed speed of 4 rotations per minute (rpm) for a total of 3 min time. In the actual test, mice were placed on the rotating cylinder, and the amount of time that each animal was able to maintain its balance was recorded. The rotarod was set to accelerate in a linear manner from 4 to 40 rpm over a 5 min time period. Time spent on the rotating rod was taken as a measure of motor coordination and balance and averaged over two trials/test day.

5 RESULTS AND DISCUSSION

5.1 Paper I and Manuscript I

SNAP-25 is critical for stimulus-evoked synaptic transmission, as neurons lacking this molecule exhibit abolished evoked neurotransmission at both neuromuscular junction and central synapses[202]. Reductions in expression levels of SNAP-25 have been associated with impairments in short-term synaptic plasticity in neuronal cultures from SNAP-25 heterozygous mice[203]. SNAP-25 is expressed both pre- and postsynaptically[204], and, while these studies help explain the presynaptic contribution of SNAP-25 in synaptic transmission, other studies have reported involvement of SNAP-25 in the surface expression of NMDA receptors postsynaptically as well[205,206]. Knocking down SNAP-25 through shRNAs impairs LTP in CA1 hippocampal synapses[206]. SNAP-25 is also associated with spine morphogenesis and structural changes, which are necessary for LTP induction and maintenance[207,208]. The opposing forms of synaptic plasticity, LTD and LTP, also undergo developmental regulation themselves, with LTD dominant early in life, slowly being taken over by LTP during development[52]. Similarly, there is evidence of developmental up-regulation of different NMDA receptors subunits expression at central synapses[209-211]. The timing of the switches in developmental regulation of synaptic LTD and LTP and NMDA receptor expression coincides with the developmental switch from SNAP-25a to SNAP-25b, suggesting that these processes might be mediated or influenced by different isoforms of SNAP-25. Our group has previously shown that short-term plasticity is impaired in young SNAP-25b-deficient mice[186], but nothing was known about how SNAP-25a and SNAP-25b contribute to the induction and expression of long-term activity-dependent plastic changes at central synapses. Utilizing a unique mouse model expressing only SNAP-25a (MT or SNAP-25b-deficient mice), and electrophysiological field recordings in brain slices from this mouse and WT littermates, we evaluated the roles of individual isoforms of SNAP-25 in different forms activity-dependent synaptic plasticity at Schaffer collateral-CA1 synapses in the hippocampus.

5.2 Alternative splicing switch from SNAP-25a to SNAP-25b is delayed in females

In Paper I, we show that the alternative splicing switch from SNAP-25a to SNAP-25b is delayed in WT females as compared to WT males in the hippocampus. At 4 weeks of age, WT females had approximately 50% SNAP-25a expression levels compared to less than 20% in WT males. Both SNAP-25b-deficient males and female mice had significantly higher SNAP-25a expression levels and no SNAP-25b, which serves to validate the mouse model, as SNAP-25b should be

completely replaced by SNAP-25a. SNAP-25b-deficient females also had significantly reduced expression levels of VAMP2. No differences were observed in the expression levels of syntaxin 1A and the postsynaptic SNARE, SNAP-47 in either SNAP-25b-deficient males or females in the hippocampus.

5.3 Reduced magnitude of LTP and enhanced LTD in the SNAP-25b-deficient mice

SNAP-25b-deficient mice exhibited significantly reduced magnitude of activity-dependent LTP at Schaffer collateral-CA1 hippocampal synapses at 4 and 8 weeks of age. By 16 weeks of age, however, the SNAP-25b-deficient mice appeared to have over-compensated for the deficit and exhibited enhanced LTP compared to WT littermates. SNAP-25b-deficient mice exhibited enhanced LTD at 4 weeks of age, but no differences in LTD at 16 weeks of age.

To probe the underlying mechanisms of these changes, we induced LTD in SNAP-25b-deficient brain slices via bath application of NMDA and a mGluRII agonist, DCG-IV. No differences were detected in either NMDA or mGluRII induced LTD between SNAP-25b-deficient and WT brain slices. It was also interesting to observe that the magnitude of LTP varied between WT males and WT females at 4 and 8 weeks of age as well. At 4 weeks of age, WT males had significantly higher magnitude of LTP compared to females but this effect was reversed at 8 weeks of age, with females having a significantly higher magnitude of LTP.

Interestingly, hippocampal-dependent learning and memory assessment in an active avoidance task altered the effects of SNAP-25b-deficiency on LTP, compared to WT littermates. LTP was enhanced at 4 weeks of age in SNAP-25b-deficient mice, while no difference in LTP was observed at 16 weeks of age, following training in the active avoidance task. Similarly, LTD was enhanced both at 4- and 16 weeks of age in SNAP-25b-deficient mice after the active avoidance training. This suggests both that learning acquisition can shift the thresholds for the induction of both LTP and LTD at Schaffer collateral-CA1 synapses, and that this dynamic regulation of the thresholds for long-term plasticity is affected by the presence of SNAP-25b.

These results indicate a clear role for the SNAP-25 isoforms in regulating long-term activity-dependent plastic changes at central synapses. In the absence of SNAP-25b, LTD dominates early in development, but after neurons have formed connections and the need for the stronger synaptic connections (synaptic maturation) arises with growth, the developmental switch to SNAP-25b serves that function. SNAP-25 is present both pre- and postsynaptically,

so these changes are likely mediated from both the loci; however, the current studies did not investigated the possible postsynaptic contributions of SNAP-25a and SNAP-25b to synaptic plasticity.

5.4 Presynaptic contributions of SNAP-25 isoforms in synaptic plasticity

Short-term plasticity (STP) assessed through paired-pulse facilitation (PPF) at various intervals is a reliable measure of presynaptic components of synaptic plasticity. We measured PPF under basal conditions and after the induction of LTP. There were no differences observed in PPF between WT and SNAP-25b-deficient mice before and after LTP; however, it was interesting to observe that in WT male mice, PPF was significantly smaller after the induction of LTP, whereas in WT females it was not, suggesting an increase in release probability in male WT mice. WT females exhibited significantly more SNAP-25a and a reduced magnitude of LTP compared to WT males, and PPF shifts coincided with those findings. However, surprisingly, in both SNAP-25b-deficient males and females, PPF became significantly smaller after the induction of LTP, also consistent with an increase in release probability. We measured the direct release kinetics of neurotransmitter release through destaining of FM1-43 (a vesicle specific dye), and found that, in SNAP-25b-deficient synapses, FM1-43 release kinetics were significantly faster than in WT mice. Faster presynaptic release kinetics reflects a higher probability of release (*Pr*) which is associated with LTP, and SNAP-25b-deficent mice exhibited deficits in LTP at similar ages. Sørenson and colleagues have shown that SNAP-25b is capable of priming a larger pool of neurotransmitter containing vesicles compared to SNAP-25a[140]. Hence, the faster release kinetics observed in FM1-43 destaining experiment might be a result of a smaller pool of vesicles in SNAP-25b-deficient mice. We conclude that SNAP-25b primes a large pool of readily releasable vesicles and, therefore, supports enhanced LTP, and the lack of SNAP-25b favors larger LTD.

Input-output relations are a measure of how effectively a synapse can translate a presynaptic stimulus into a postsynaptic response. This reflects the basal strength of a synapse and can affect, among other things, the induction threshold for synaptic plasticity. Input-output curves were significantly steeper in WT mice after the induction of LTP, compared to pre-tetanus baselines. However, in SNAP-25b-deficient mice, these curves did not differ for the initial current increments after the induction of LTP, but did become significantly steeper afterwards and did not reach saturation, like in WT mice. There was no difference in SNAP-25b-deficient and WT mice input-output curve under basal conditions. However, after the

induction of LTP, SNAP-25b-deficient mice showed significantly larger increases in the ceiling response amplitude.

Taken together, these results demonstrate that SNAP-25a and SNAP-25b perform independent functions at the presynaptic terminal at different stages of development by directly regulating the readily releasable pool of neurotransmitter containing vesicles, which eventually affects the induction of synaptic plasticity at Schaffer collateral-CA1 synapses in the hippocampus.

5.5 Abnormalities of synaptic transmission associated with deficits in learning and memory formation

To assess if the abnormalities in synaptic transmission (STP, LTP and LTD) in SNAP-25b-deficient mice were associated with deficits in learning acquisition and memory formation, we employed an active avoidance learning task. In this test, mice had to learn the location of a shock zone on a rotating grid using external visual cues, and remember it in order to avoid being shocked as the grid moved the animal into the shock quadrant. Then the shock zone was turned off (extinction of memory) and, in the following testing session, a new shock zone was assigned, so the mice had exhibit behavioral flexibility to learn the new shock zone position. At 4 weeks of age, SNAP-25b-deficient mice were slow to learn the initial shock zone but, after extinction, they learned the new shock zone faster than their WT littermates. This suggests that SNAP-25b-deficient mice formed a weaker memory of the initial shock zone, which made the extinction and re-learning of the new shock zone relatively easier.

These results are consistent with the observed deficits in LTP/enhanced LTD at 4 weeks of age. At 16 weeks of age, there were no differences observed between SNAP-25b-deficient and WT mice in the initial learning of the shock zone, but relearning after extinction was faster in SNAP-25b-deficient mice compared to WT littermates, consistent with the compensatory changes in LTP observed at 16 weeks of age.

We also tested SNAP-25b-deficient mice for anxiety-like behavior, using an elevated plus maze at 4 and 16 weeks of age. At 4 weeks of age, SNAP-25b-deficient mice exhibited a greater tendency to remain in the closed arm of the maze, suggesting higher levels of anxiety than WT mice, but no differences were observed at 16 weeks of age compared to WT littermates.

These behavioral assessments are consistent with our *ex vivo* LTP and LTD findings, associating stronger LTP in the presence of SNAP-25b with stronger learning and memory formation.

5.6 Paper II

Gβγ subunits from the activated GPCRs are known to inhibit exocytosis via interacting with the C-terminus of SNAP-25 presynaptically by competing with synaptotagmin 1 in a Ca^{2+} dependent manner. Gβγ subunits are released intracellularly when GPCRs are activated on the cell surface, and these subunits can interact directly with the trimeric SNARE core complexes, as well as with the individual SNARE proteins inhibiting exocytosis. The nature of the negative interaction between Gβγ subunits and the C-terminus of SNAP-25 have been validated in a number of *in vitro* studies, but how this interaction can affect the induction and expression of long-term activity dependent plastic changes at central synapses, was not known. Therefore, we utilized another mouse model to investigate this question. This gene targeted mouse was developed by Prof. Heidi Hamm and colleagues at Vanderbilt University. In these mice, the interaction between Gβγ subunits and the C-terminus of SNAP-25 was genetically reduced by two-folds without affecting the SNAP-25-synaptotagmin 1 interaction. Introduction of the $G204^{*}$ mutation in the extreme C-terminus of SNAP-25 and mutating the three amino acids (glycine, serine, glycine) via CRISPR-Cas9 strategy resulted in the SNAP-25Δ3 mouse model[179].

5.7 A SNAP-25 Δ3 mouse model with disrupted binding to Gβγ exhibited enhanced LTP at Schaffer collateral-CA1 synapses in hippocampal slices

Electrophysiological field recordings in brain slices from SNAP-25Δ3 mice revealed enhanced activity-dependent LTP at Schaffer collateral-CA1 synapses compared to WT mice. This finding suggests that Gβγ-SNAP-25 interaction is an important negative regulatory mechanism controlling the magnitude of LTP induction and expression. Further investigations are, required to assess the role of Gβγ-SNAP-25 interaction in STP and LTD as well. It will also be interesting to evaluate these SNAP-25Δ3 mice in the behavioral assay of active avoidance, as they have been recently reported to exhibit defective spatial learning in the Morris water maze task, along with increased stress-induced hyperthermia, impaired gait, and supraspinal nociception[179].

5.8 Manuscript II

The final study included in this thesis pursued the possible common underlying pathophysiological mechanisms between metabolic syndrome and cognitive impairment. Cognitive impairment is a common symptom observed in the progression of neurodegenerative diseases, for example, Alzheimer disease (AD) and related dementias[212]. The linking feature between cognitive impairment and metabolic syndrome is often considered to be insulin resistance in the brain[213-215]. In support of this claim, administration of intranasal insulin has been shown to markedly improve cognition in AD patients[216]. In addition, the metabolic syndrome induced by high-fat/high-sugar diet (WD) has been shown to affect brain signaling pathways in different brain regions[217,218], affecting cognition and causing anxiety and depressive like behaviors[219]. Our group has previously shown that replacing SNAP-25b with SNAP-25a in a mouse model results in a diabetic phenotype characterized by obesity, impairments in lipid and glucose homeostasis, liver steatosis and hypothalamic dysfunction, conditions exacerbated dramatically when combined with Western diet[197]. SNAP-25 is a key SNARE protein involved in the release of neurotransmitters and hormones and therefore, in this study, we investigated if SNAP-25b-deficient mice with metabolic syndrome showed signs of cognitive impairment, and what might be the underlying causes for those impairments.

5.9 SNAP-25b-deficient mice with metabolic syndrome exhibited cognitive impairments on various behavioral paradigms

We tested SNAP-25b-deficient mice with metabolic syndrome after going through a 7-week WD intervention in hippocampal dependent 'spatial object location task', prefrontal cortex dependent 'forced swim test' and cerebellum dependent 'rotarod test'. The experimental design consisted of similar SNAP-25b-deficient mice on control diet (CD) and WT mice on CD and WD for comparison as well. Both male and female SNAP-25b-deficient mice both on CD and WD exhibited impaired performance on spatial object location task compared to WT mice on CD. Similarly, SNAP-25b-deficient mice on CD and WD exhibited depressive-like behavior in the forced swim test, except SNAP-25b-deficient female mice on WD compared to WT mice on CD. In the rotarod test, both WD and SNAP-25b deficiency appeared to have affected the performance, as all the experimental groups exhibited impaired performance compared to WT CD mice. These findings strengthen the notion that metabolic syndrome affects higher cognitive functions of the brain and SNAP-25 mediated altered hormonal and

neurotransmitter release could possibly be a contributing, common pathophysiological mechanism.

5.10 Brain regions specific SNARE proteins expression

Next, we quantified the SNARE proteins SNAP-25, syntaxin 1A, VAMP2 and SNAP-47 in the hippocampus, prefrontal cortex and cerebellum of the mice, which underwent behavioral testing. Unlike the behavioral tests results, in which males and females did not differ much, we observed differences in the expression profiles of these proteins between male and female mice. SNAP-25 expression levels were found to be significantly upregulated in the hippocampi of female SNAP-25b-deficient mice on CD and WD but not in males. Syntaxin 1A and VAMP2 levels were found to be significantly upregulated in the hippocampi of SNAP-25b-deficient male and female mice on WD, but syntaxin 1A and VAMP2 levels were also significantly up-regulated in SNAP-25b-deficient female mice on CD but not in males. In the prefrontal cortex, SNAP-25 expression levels were significantly upregulated in the SNAP-25b-deficient male mice on WD, but only in SNAP-25b-deficient female mice on CD. Syntaxin 1A expression levels were significantly upregulated in SNAP-25b-deficient male mice both on CD and WD, but only in female SNAP-25b-deficient mice on CD. VAMP2 expression levels were significantly upregulated in the SNAP-25b-deficient male mice both on CD and WD but not in female SNAP-25b-deficient mice. SNAP-47 levels were significantly upregulated in the prefrontal cortex primarily in response to WD, as both WT and SNAP-25b-deficient mice on WD exhibited elevated levels of SNAP-47 in both male and female mice. In cerebellum, SNAP-25 and syntaxin 1A levels were found to be significantly upregulated only in the SNAP-25b-deficient male mice on WD. VAMP 2 levels were significantly up-regulated only in SNAP-25b-deficient female mice on CD. SNAP-47 levels in the cerebellum followed a similar trend as in prefrontal cortex and were found to be elevated primarily in response to WD in both male and female, WT and SNAP-25b-deficient mice. These results indicate that metabolic syndrome caused by the lack of SNAP-25b and/or WD affects SNARE proteins expression levels in a sex dependent manner and also impairs cognition, motor coordination and induce depressive like behavior.

5.11 Conclusion

SNAP-25 plays a central role in synaptic transmission at synapses in the central nervous system. It regulates neurotransmitters release presynaptically, and interacts with ancillary proteins, which altogether control important aspects of activity-dependent long-term

synaptic plasticity. The two developmentally regulated isoforms of SNAP-25, SNAP-25a and SNAP-25b, differ in their abilities to perform these functions and, depending on which isoform is present, physiology of synaptic transmission can differ significantly. LTD is pre-dominant early in development, but is compensated as development proceeds by enhanced expression of LTP. This apparent switch from LTD to LTP coincides temporally with the switch from SNAP-25a to SNAP-25b in the hippocampus. We show here that, in the absence of SNAP-25b, when only SNAP-25a supports presynaptic glutamatergic function, LTP is weaker and LTD is stronger. These changes in synaptic plasticity also correlate with up-regulation of the capacity for learning acquisition and memory formation with growth. We also show here that weaker LTP and stronger LTD were observed in parallel with deficiencies in learning acquisition and memory formation in a behavioral spatial learning assay in the mutant mouse lacking SNAP-25b.

While our data suggest a key role for SNAP-25 isoform switching in regulating presynaptic function and synaptic plasticity, the roles of SNAP-25 isoforms in postsynaptic receptor trafficking still need further investigation. Furthermore, we also have evidence of sex differences, in that males and females do not follow the same timeline for the alternative splicing switch from SNAP-25a to SNAP-25b in the hippocampus, with the switch delayed in females. Interaction between the C-terminus of SNAP-25 and Gβγ is an important determinant of presynaptic inhibition, and the induction of LTD at central synapses[181], and reducing this interaction in gene targeted mice expressing SNAP-25 that lacks the 3 c-terminus amino acids, resulted in enhanced LTP. Finally, we have shown that common pathophysiological mechanisms likely are mediated via SNARE-related mechanisms, suggesting the importance of studying comorbidities, such as metabolic diseases like type 2 diabetes mellitus, which involves abnormal insulin secretion and insulin resistance, along with neurological diseases associated with cognitive impairment.

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